Transgenic Res (2007) 16:479–489
DOI 10.1007/s11248-007-9093-z

ORIGINAL PAPER

# Insulin-like growth factor I (IGF-I) in a growth-enhanced transgenic (GH-overexpressing) bony fish, the tilapia (*Oreochromis niloticus*): indication for a higher impact of autocrine/paracrine than of endocrine IGF-I

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Received: 1 December 2006 / Accepted: 19 March 2007 / Published online: 13 April 2007


**Abstract** Several lines of growth hormone (GH)-overexpressing fish have been produced and analysed for growth and fertility parameters. However, only few data are available on the growth-promoting hormone insulin-like growth factor I (IGF-I) that mediates most effects of GH, and these are contradictory. Using quantitative real-time RT-PCR, radioimmunoassay, in situ hybridization, immunohistochemistry, and radiochromatography we investigated IGF-I and IGF binding proteins (IGFBPs) in an adult (17 months old) transgenic (GH-overexpressing) tilapia (*Oreochromis niloticus*). The transgenics showed an around 1.5-fold increase in length and an approximately 2.3-fold higher weight than the non-transgenics. Using radioimmunoassay, the serum IGF-I levels were lower (6.22 ± 0.75 ng/ml) in transgenic than in wild-type (15.01 ± 1.49 ng/ml) individuals ($P = 0.0012$). Radioimmunoassayable IGF-I in transgenic liver was 4.2-times higher than in wild-type (16.0 ± 2.21 vs. 3.83 ± 0.71 ng/g, $P = 0.0017$). No hepatocytes in wild-type but numerous hepatocytes in transgenic liver contained IGF-I-immunoreactivity. RT-PCR revealed a 1.4-times higher IGF-I mRNA expression in the liver of the transgenics (10.51 ± 0.82 vs. 7.3 ± 0.49 pg/μg total RNA, $P = 0.0032$). In correspondence, in situ hybridization showed more IGF-I mRNA containing hepatocytes in the transgenics. A twofold elevated IGF-I mRNA expression was determined in the skeletal muscle of transgenics (0.33 ± 0.02 vs. 0.16 ± 0.01 pg/μg total RNA, $P < 0.0001$). Both liver and serum of transgenics showed increased IGF-I binding. The increased IGFBP content in the liver may lead to retention of IGF-I, and/or the release of IGF-I into the circulation may be slower resulting in accumulation of IGF-I in the hepatocytes. Our results indicate that the enhanced growth of the transgenics likely is due to enhanced autocrine/paracrine action of IGF-I in extrahepatic sites, as shown here for skeletal muscle.

**Keywords** IGF-I · IGFBP · Liver · Serum · Skeletal muscle · Transgenic fish

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## Introduction

Insulin-like growth factor I (IGF-I) is a potent mitogenic hormone that induces growth and differentiation in a variety of target organs (Jones and Clemmons 1995; Reinecke and Collet 1998; Butler and LeRoith 2001). In mammals, IGF-I is mainly

produced in the liver which is the principal source of endocrine IGF-I. The primary stimulus for the synthesis and secretion of liver IGF-I is growth hormone (GH) released from the anterior pituitary (Reinecke et al. 2005).

Like in mammals, the major site of IGF-I gene expression in bony fish is liver (Duan 1998; Reinecke et al. 2005; Wood et al. 2005). High-affinity GH binding sites have been characterized in salmon and tilapia liver (Gray et al. 1992; Ng et al. 1992; Shepherd et al. 1997). In accordance, GH injections promoted liver IGF-I mRNA expression in salmonids and tilapia (e.g. Duan et al. 1993a; Moriyama 1995; Shamblott et al. 1995; Duguay et al. 1996; Shepherd et al. 1997; Guillén et al. 1998; Kajimura et al. 2001; Vong et al. 2003) and raised IGF-I plasma levels in salmonids, seabream and tilapia (e.g. Moriyama 1995; Shamblott et al. 1995; Guillén et al. 1998; Moriyama et al. 2000; Kajimura et al. 2001), which was accompanied by an increase in growth (Guillén et al. 1998). Similarly, GH also stimulates the expression of IGF-I mRNA in primary hepatocyte cultures of bony fish, such as salmonids (Duan et al. 1993a, b; Shamblott et al. 1995; Pierce et al. 2005) and tilapia *Oreochromis mossambicus* (Schmid et al. 2000). In adult Coho salmon, continuous infusion of bovine IGF-I increased growth rate and weight gain (McCormick et al. 1992), and plasma IGF-I levels and growth rate were significantly correlated in *O. mossambicus* (Kajimura et al. 2001; Uchida et al. 2003).

Several groups have produced different lines of transgenic fish (e.g. Devlin et al. 2000; Maclean et al. 2002; Rocha et al. 2004). Among the transgenic fish those carrying exogenous GH gene constructs constitute the majority. The obvious reason for this focus of interest is to improve fish growth in aquacultural food production (Zbikowska 2003). Mainly salmonids (Du et al. 1992; Devlin et al. 1994, 2000; Sundstrom et al. 2004), carp (Zhang et al. 1990), tilapia (Martinez et al. 1996; Rahman et al. 1998), and sea bream (Lu et al. 2002) have been used for genetically induced growth enhancement. On the other hand, GH-overexpressing fish are also considered as excellent models to study gene regulation and development (Amsterdam and Becker 2005). However, investigations on transgenic fish have almost exclusively dealt with growth parameters, fertility, and body and organ integrity (Maclean et al. 2002; Sundstrom et al. 2004).

The above referred studies demonstrate the stimulating action of GH on IGF-I-mediated growth in bony fish either by bolus injections of GH or by adding GH to the medium of primary hepatocyte cultures and, thus, deal with short-term effects of GH. In contrast, only one study has dealt with IGF-I in transgenic GH-overexpressing fish. In Coho salmon *Oncorhynchus kisutch* IGF-I serum levels were determined and gave contradictory results (Devlin et al. 2000): in one experiment they were slightly enhanced and in another slightly reduced. In order to investigate whether the severe enhancement in body growth in GH-overexpressing fish may be due to endocrine (liver-derived) IGF-I and/or enhanced IGF-I expression in skeletal muscle we used a transgenic GH-overexpressing and age-matched wild-type tilapia (*Oreochromis niloticus*). The following parameters were analysed: (1) the expression level of IGF-I mRNA in liver and skeletal muscle using quantitative real-time PCR, (2) the concentration of IGF-I peptide in liver and serum by RIA, (3) the localization of IGF-I in liver at the mRNA level by in situ hybridization and at the peptide level by immunohistochemistry, and (4) the IGF-I binding proteins (IGFBPs) in liver and serum by radiochromatography.

## Material and methods

### Animals

The transgenic fish used in these experiments have been previously produced from crosses between a wild-type female *O. niloticus* and a G1 transgenic male. This line of growth-enhanced tilapia (C86) carries a single copy of a Chinook salmon *Oncorhynchus tshawytscha* GH gene spliced to an ocean pout *Macrozoarces americanus* antifreeze promoter (OP-AFPcsGH) co-ligated with a carp beta actin/lacZ reporter gene construct, integrated into the tilapia genome (Rahman et al. 1998). Tilapia of the C86 strain and the non-transgenic siblings were bred at the University of Southampton. The fish were kept in fresh water tanks at 27 ± 1°C under a 12 h/12 h light/dark cycle and fed to satiation. Adult (17 months)

transgenic (TG) and wild-type (WT) fish were used for the experiments. Fish were anaesthetized with 2-phenoxy-ethanol (Sigma, St Louis, MO, USA) added to water, measured in weight and length, and the tissue samples excised. Principles of animal care and specific national laws were followed.

### Detection of transgene

The transgenic or non-transgenic state of the individuals was detected by RT-PCR followed up by Southern Blotting. For PCR amplification an approximately 3 mm × 2 mm fin clip was taken from the caudal fin and immediately frozen in liquid nitrogen. Genomic DNA was isolated. The standard procedures for isolation and purification of DNA (Rahman and Maclean 1992) and subsequent PCR and Southern Blotting were performed as described before (Rahman et al. 1998).

### Tissue sampling and extraction

Liver ($n$ = 10 TG, $n$ = 10 WT) and skeletal muscle ($n$ = 6 TG, $n$ = 6 WT) specimens for RT-PCR were excised and immediately transferred into 1.5 ml of the RNA-preserving reagent RNAlater™ (Ambion, Austin, USA). The samples were kept at 4°C to promote inactivation of RNAses and later stored at −20°C until RNA isolation. Total RNA was extracted using TRIzol reagent (GibcoBRL), treated with 1 U of RQ1 RNAse-free DNase (Catalys AG, Wallisellen, Switzerland), resuspended in DEPC-treated $H_2O$ and stored at −80°C.

### Absolute quantification of liver and skeletal muscle IGF-I gene expression by real-time PCR

Absolute quantification of liver and skeletal muscle IGF-I mRNA was performed as already described (Caelers et al. 2004). In brief, based on the gene sequences of *O. mossambicus* IGF-I (Reinecke et al. 1997), and *O. niloticus* $\beta$-actin as a housekeeping gene (Hwang et al. 2003), tilapia specific primers and probes for real-time RT-PCR were designed with the Primer Express software version 1.5 (PE Biosystems, Foster City, CA, USA). To create templates for in vitro transcription, a T7 phage polymerase promoter gene sequence was added by primer extension to the 5′end of the antisense primers using conventional RT-PCR of *O. niloticus* liver total RNA. For cDNA synthesis, 5 µg RNA were annealed with 1 µM poly(dT) primer (5′-CCTGAATTCTAGAGCTCAT(dT17)-3′) for 5 min at 70°C. The RNA/primer mix was incubated for 1 h at 37°C with 10 mM dNTPs and 100 U M-MLV-RTase (Promega) in 1× buffer. One microlitre aliquots of cDNA were added to 50 µl PCR reaction using the Thermo-Start™ PCR Master Mix (Abgene, NY, USA). Amplification conditions were optimized for a Stratagene RoboCycler Gradient 40: 1 cycle 10 min at 94°C, 1 min at 60°C, 2 min at 72°C; 40 cycles 1 min at 94°C, 1 min at 60°C, 2 min at 72°C; final extension 10 min at 72°C. PCR products including the T7 promoter gene sequence were sequenced and visualized on a 2.5% agarose gel. Standard cRNAs were generated by in vitro transcription using the T7-MEGAshortscript™ Kit (Ambion), analysed by photospectrometry and UV-shadowing, and quantified by photospectrometry and dot blot. Defined amounts at tenfold dilutions were subjected to real-time PCR using a one-Step-RT-PCR Mastermix (Applied Biosystems, Rotkreuz, Switzerland). RT-step (48°C, 30 min) and denaturation step (95°C, 10 min) were performed followed by 40 cycles (95°C, 15 s; 60°C, 1 min) in a single tube using ABI PRISM™ 7700 Sequence Detection System Perkin Elmer (Applied Biosystems). Standard curves were generated based on the linear relationship between $C_T$ value and logarithm of the starting amount. For measurements, tenfold diluted defined amounts of standard cRNA and 10 ng of total RNA were subjected in parallel to real-time PCR under the same experimental settings. To calculate absolute amounts the different lengths of cRNA and mRNA were considered by a correction factor determined by division of lengths of IGF-I mRNA and cRNA (546 nt/70 nt = 7.8).

### Peptide extraction of serum and liver

Blood ($n$ = 5 TG, $n$ = 5 WT) was taken with a 1 ml heparinized syringe from the caudal vein and collected in sterile 0.5 ml tubes. The blood was centrifuged at 13,000 rpm for 15 min at 4°C and the serum removed and stored at –20°C until use. 1 g of liver ($n$ = 5 TG, $n$ = 5 WT) was homogenized with 5 ml 0.1 M sodium phosphate buffer (pH 7.0) and centrifuged at 10,000$g$ for 15 min at 4°C. The supernatant was removed with a Pasteur pipette,

deep-frozen immediately, and stored at -20°C until use. To dissociate IGF-I from the binding proteins, acid–ethanol extraction was performed as already described. In brief, 40 µl of serum and liver homogenate, respectively, was thoroughly mixed with 160 µl of an acid–ethanol extraction mix (87.5% ethanol, 12.5% 2 M HCl, v/v) and incubated for 30 min at room temperature. After neutralization with 0.885 M Tris base, the extracts were centrifuged at 13,000 rpm at 4°C for 10 min. 50 µl of the supernatant was used for the IGF-I radioimmunoassay.

### Radioimmunoassay in serum and liver

Serum and liver IGF-I was measured using the Fish IGF-I RIA kit for the tilapia *O. mossambicus* (GroPep Pty Ltd., Adelaide, Australia) with recombinant tilapia IGF-I as tracer and standard according to the protocol of the manufacturer.

### Radiochromatography for IGFBPs in serum and liver

Radiochromatography was performed as described earlier (Zapf et al. 1975, 1989). Briefly, 0.2 ml of pooled serum or liver homogenate was diluted with 0.3 ml of Dulbecco's phosphate-buffered saline, pH 7.4, containing $NaN_3$ and 500 U of Trasylol (Bayer, Germany), and incubated for 24 h at 4°C with $^{125}$I-labelled tilapia IGF-I ($2.5 \times 10^5$ CPM, GroPep Ltd.). The mixture was run through a Sephadex G-200 column (2.1 cm × 70 cm) preequilibrated with Dulbecco's buffered saline, pH 7.4, and the radioactivity of the collected fractions (2.5 ml) was counted in a gamma-counter.

### Preparation of probes for in situ hybridization

Probes were prepared as described (Schmid et al. 1999; Berishvili et al. 2006a, b). In brief, total RNA from tilapia liver was extracted with the Ultraspec Extraction Kit (ams, Lugano, Switzerland). For cDNA synthesis 5 µg RNA was annealed with 1 µM of a poly(dT) primer (5′ CCTGAATTCTAGAGCTCAT(dT17) 3′) for 3 min at 70°C. The RNA/primer mix was incubated for 1 h at 37°C with 15 mM dNTPs and 10 U AMV-RTase (Pharmacia, Switzerland) in 1× reaction buffer (50 mM Tris–HCl/pH 8.3, 40 mM KCl, 6 mM $MgCl_2$). One microlitre cDNA was incubated with 1 µM of sense (5′-GTCTGTGGAGAGCGAGGCTTT-3′) and antisense (5′-AACCTTGGGTGCTCTTGGCATG-3′) primers corresponding to the B- and E-domain, 200 µM dNTPs, and 1 U Taq-polymerase (Pharmacia) in 1× incubation buffer (10 mM Tris–HCl/pH 8, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001 % gelatine). The amplification program was optimized for a Stratagene RoboCycler Gradient 40:1 cycle 10 min at 94°C, 1 min at 59°C, 2 min at 72°C; 30 cycles 1 min at 94°C, 1 min at 59°C and 2 min at 72°C followed by final extension for 5 min at 72°C. PCR fragments were separated on a 2% agarose gel and eluted by the Gel Extraction Kit QIAquick (Qiagen, Switzerland). Thereafter, the PCR products were cloned in a pCR-Script SK(+) cloning vector using a kit (Stratagene, Heidelberg, Germany). Plasmids containing the gene sequence fragments were sequenced (Microsynth, Switzerland) and the sequences compared to database. The plasmids containing the specific inserts of IGF-I (207 bp) were used as templates for the synthesis of digoxigenin (DIG)-labelled RNA probes. Linearization was performed with *EcoRI* for T3- and *NotI* for T7-polymerase-driven transcription. One microgram of linearized plasmids was transcribed in vitro in the presence of DIG-UTP from T3 and T7 promotors to obtain antisense and sense (negative control) probes. Integrity of probes and efficiency of labelling were confirmed by dot blot and gel electrophoresis including blotting and incubation with antibody. Specificity of the probes has been demonstrated previously (Schmid et al. 1999; Berishvili et al. 2006a).

### In situ hybridization

Liver preparations ($n$ = 3 WT, $n$ = 3 TG) were fixed by immersion with 4% buffered formalin for 4 h at room temperature. Specimens were dehydrated in ascending series of ethanol and routinely embedded in paraplast (58°C). Four micrometre sections were mounted on Super Frost Plus slides (Menzel-Gläser, Germany) and dried overnight at 42°C. After dewaxing and rehydration, the sections were postfixed with 4% paraformaldehyde and 0.1% glutaraldehyde in 1× PBS. In situ hybridization was performed as previously described (Schmid et al. 1999; Berishvili et al. 2006a, b). The following steps were carried out with DEPC-treated solutions in a humified chamber. The

sections were digested with 0.02% proteinase K in 20 mM Tris–HCl/pH 7.4, 2 mM $CaCl_2$ for 10 min at 37°C. Thereafter, sections were treated with 1.5% triethanolamine and 0.25% acetic anhydride for 10 min at room temperature and incubated with 50 µl prehybridization solution per section for 3–4 h at 54°C. Hybridization was performed overnight at 54°C with 30 µl of hybridization buffer containing 10 ng of sense or antisense probe previously denaturated for 5 min at 85°C. Slides were washed for 15 min at room temperature in 2× SSC, and for 30 min at the specific hybridization temperature at descending concentrations of SSC (2×, 1×, 0.5×, 0.2×). The alkaline-phosphatase-coupled anti-DIG antibody was diluted 1:4,000 in 1% blocking reagent (Roche-Diagnostics) in buffer P1 and sections were incubated for 1 h at room temperature in the dark. After washing twice in P1 for 15 min, sections were treated with buffer P3, 5 mM levamisole, and NBT/BCIP stock solution (Roche Diagnostics). Colour development was carried out overnight at room temperature and stopped by rinse in tap water for 15 min. Sections were mounted with glycergel and photographed with a Zeiss Axioscope using the Axiovision software 3.1. (Zeiss, Zürich, Switzerland).

### Immunohistochemical technique

Specimens for immunohistochemistry ($n$ = 3 WT, $n$ = 3 TG) were immersed in Bouin's solution without acetic acid for 4 h at room temperature. Specimens were dehydrated in ascending series of ethanol and routinely embedded in paraplast (58°C). Sections were cut at 4 µm, mounted onto glass slides and dried overnight at 42°C. After dewaxing and rehydration unspecific binding was reduced by treatment with PBS (pH 7.4) containing 2% bovine serum albumine for 30 min at room temperature. Thereafter, the sections were incubated overnight with the rabbit antiserum 116 raised against human IGF-I (1:400) and washed repetitively in PBS. This antiserum specifically also stains IGF-I in fish as shown by absorption experiments (Reinecke et al. 1997; Schmid et al. 1999; Berishvili et al. 2006a, b). The IGF-I antiserum was detected by incubation with biotinylated goat anti-rabbit IgG (Bioscience Products, Emmenbrücke, Switzerland, 1:100) for 30 min at room temperature. After repetitive rinsing in PBS, the reactions were visualized with fluorescein-isothiocyanate-conjugated streptavidin (Bioscience Products, 1:100) for 30 min at room temperature in the dark. Sections were mounted with glycergel and photographed with a Zeiss Axioscope using the Axiovision software 3.1.

### Statistical analysis

Statistical analysis of the data was performed with a GraphPad Prism 4 program. This included a Mann–Whitney-$U$-test with a significance level of 5%. All data are expressed as means ± SEM.

## Results

### Fish size and weight

The 17-month old transgenic fish showed an approximately 1.5-fold increase in head–tail length when compared to their non-transgenic siblings (28.7 ± 8.3 vs. 18.5 ± 6.2 cm). The mean weights of the transgenics were around 2.3-fold higher than those of their non-transgenic siblings (415.6 ± 98 g and 184 ± 64 g, respectively).

### Measurement of IGF-I peptide and mRNA

Using radioimmunoassay, the serum IGF-I levels were lower (6.22 ± 0.75 ng/ml) in transgenic than in wild-type (15.01 ± 1.49 ng/ml) individuals ($P$ = 0.0012) (Fig. 1a). As measured by the same radioimmunoassay, the IGF-I concentration in liver amounted to 16.0 ± 2.21 ng/g in transgenics and to 3.83 ± 0.71 ng/g in wild-type animals ($P$ = 0.0017) (Fig. 1b). Real-time PCR revealed a 1.4-times higher IGF-I mRNA expression in the liver of the transgenics (10.51 ± 0.82 vs. 7.3 ± 0.49 pg/µg total RNA, $P$ = 0.0032, Fig. 1c) and a twofold elevated IGF-I mRNA expression in the skeletal muscle (0.33 ± 0.02 vs. 0.16 ± 0.01 pg/µg total RNA, $P$ < 0.0001, Fig. 1d).

### Localization of IGF-I mRNA and peptide

In situ hybridization with antisense DIG-labelled RNA probe specific for tilapia IGF-I revealed positive responses in liver whereas the sense RNA

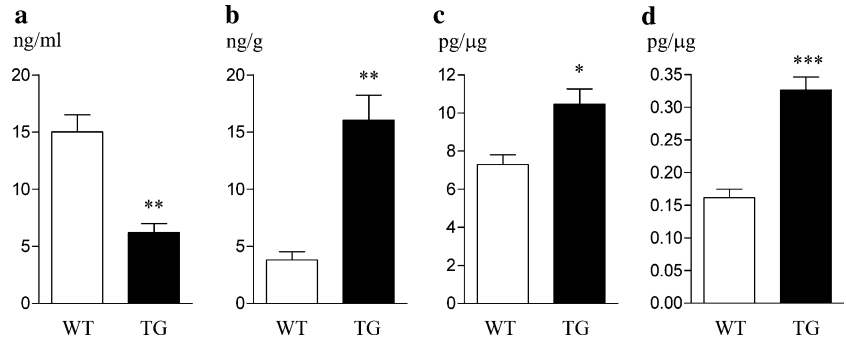


**Fig. 1** (**a**, **b**) IGF-I peptide concentrations in serum (**a**) and liver (**b**) were determined by a species-specific RIA. IGF-I mRNA in liver (**c**) and muscle (**d**) were absolutely quantified by real-time PCR. Columns give mean values of 5 (**a**, **b**), 10 (**c**) and 6 (**d**) individuals and bars give SEM. *WT* wild-type, *TG* transgenics. Significance levels in Mann–Whitney-*U*-test: $*P = 0.0032$; $**P = 0.0012$, $P = 0.0017$, $***P < 0.0001$

probe (negative control) showed no signals. In liver from non-transgenic tilapia, IGF-I mRNA occurred in numerous hepatocytes which were distributed in clusters throughout the parenchyma (Fig. 2a). Liver from transgenic individuals exhibited a higher degree of labelled cells. This was especially pronounced around the veins where numerous IGF-I mRNA containing hepatocytes were present (Fig. 2b).

In the liver of wild-type tilapia no IGF-I immunoreactivity was observed in hepatocytes (Fig. 2c). In contrast, numerous hepatocytes in transgenic liver contained IGF-I-immunoreactivity (Fig. 2d).

## Determination of IGFBPs in serum and liver

Radiochromatography of serum from wild-type and transgenic tilapia gave two main radioactive peaks around fraction 61 and fraction 85. In serum, the first peak, an IGF-binding peak corresponding to a molecular mass of 40–50 kDa, was about 1.6-fold higher in the transgenic than in the wild-type fish (Fig. 3a) as determined by planimetry. The second peak representing unbound $^{125}$I-IGF-I tracer was slightly higher for the wild-type serum, which is in line with the observed decreased binding of the tracer

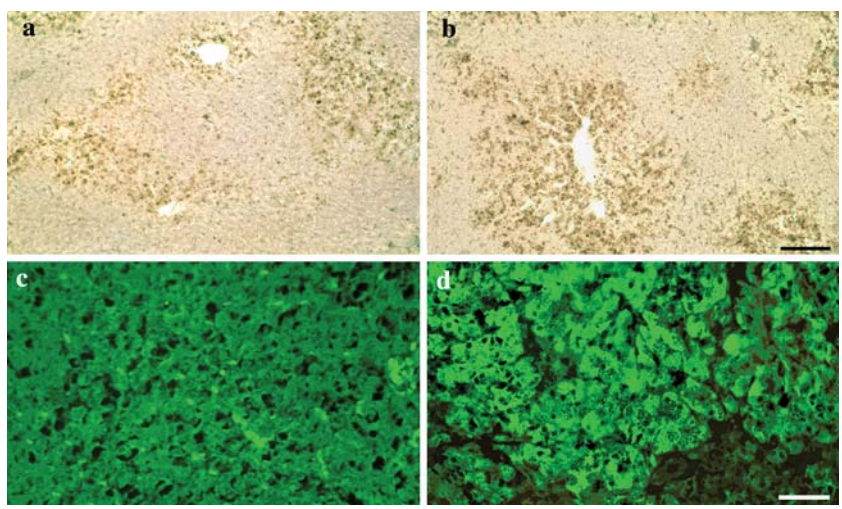


**Fig. 2** IGF-I mRNA expression (**a**, **b**) and IGF-I immunoreactivity (**c**, **d**) in wild-type (**a**, **c**) and transgenic (**b**, **d**) tilapia liver. In situ hybridization was performed with antisense DIG-labelled RNA probe specific for tilapia IGF-I. Immunofluorescence used an IGF-I specific antiserum. In liver from wild-type tilapia, IGF-I mRNA is detected in numerous hepatocytes throughout the parenchyma (**a**) but no IGF-I peptide (**c**) is found. In liver from transgenic individuals, numerous IGF-I mRNA containing hepatocytes occur (**b**) and numerous hepatocytes contain IGF-I-immunoreactivity (**d**). *Bar* (**a**, **b**) 100 µm, *bar* (**c**, **d**) 20 µm

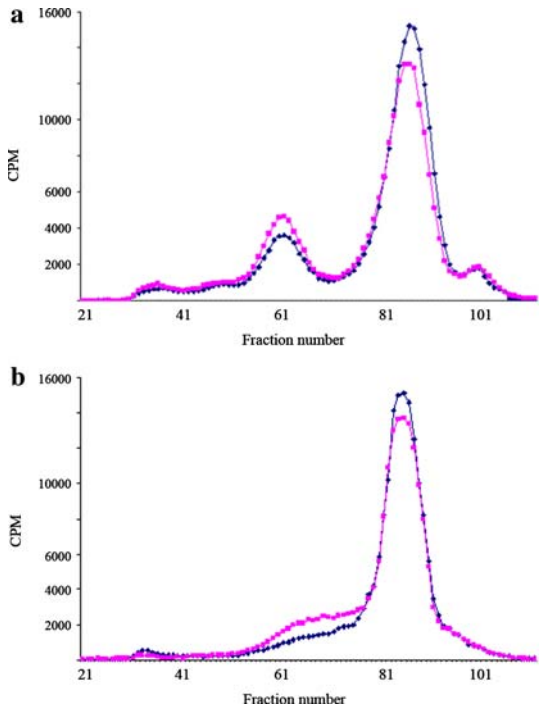


**Fig. 3** Radiochromatography of a serum and b liver homogenate from wild-type (blue curve) and transgenic (red curve) tilapia. (**a**) The peak around fraction 61 corresponds to a molecular mass of 40—50 kDa. It is preceded by two minor peaks around fractions 34 and 48. The peak around fraction 85 represents unbound 125I-IGF-I tracer. (**b**) The binding peak (''shoulder'') elutes between fractions 57 and 70. The small peak around fraction 48 observed in serum (**a**) is not present in liver (**b**). CPM counts per minute

in the wild-type fish. The 40–50 kDa binding peak disappeared when preincubation of the sera with the tracer was performed in the presence of excess cold IGF-I (not shown) indicating that binding was specific. At the same time, the peak of unbound tracer increased due to the inhibition of binding. Two minor peaks around fractions 34 and 48 preceded the 40–50 kDa binding peak (Fig. 3a). Similar to the latter, the peak around fraction 48 was displaced by cold IGF-I, but its height was not essentially different in wild-type and transgenic serum. In contrast, the radioactive peak around fraction 34 was not displaced by cold IGF-I indicating non-specific binding.

In the liver homogenate (Fig. 3b) a broader binding peak (a ''shoulder'' between fractions 57 and 70) was observed that was again higher (about 1.7-fold) in the transgenic than in the wild-type tilapia, whereas the reverse was true for the peak representing unbound tracer. Also here, binding disappeared in the presence of cold IGF-I (not shown). The small peak around fraction 48 observed in serum was not present in liver homogenate.

## Discussion

When compared to their non-transgenic siblings neither the whole phenotype nor the inner organs of the transgenic tilapia of the C86 strain used here exhibited any obvious abnormalities (Maclean et al. 2002; Caelers et al. 2005). Food conversion efficiency was about 20% better in the transgenics and these turned out to be more efficient utilizers of protein and energy (Rahman et al. 1998). This is reflected by the 2.3-fold higher mean body weight and the 1.5-fold higher mean body length of the 17-month old transgenics investigated.

According to real-time PCR and in situ hybridization IGF-I was highly expressed in wild-type tilapia liver. These results correspond to those of previous PCR and Northern blot studies (e.g. Duan et al. 1993a, b; Duguay et al. 1996; Vong et al. 2003; Caelers et al. 2004) and studies using in situ hybridization (Schmid et al. 1999; Berishvili et al. 2006b) which have also reported a high content of IGF-I mRNA in bony fish liver. Despite the expression of IGF-I mRNA, no IGF-I-immunoreactive hepatocytes could be observed. Earlier studies have also failed to detect IGF-I-immunoreactivity in bony fish hepatocytes (Richardson et al. 1995; Reinecke et al. 1997). Because the same antiserum from the latter study detects IGF-I in numerous other organs of the tilapia (Reinecke et al. 1997; Schmid et al. 1999; Berishvili et al. 2006a, b) as well as in hepatocytes of the transgenics (this study), the negative result cannot be explained by the failure of the anti-mammalian IGF-I antiserum to crossreact with fish, as discussed by Plisetskaya (1998). Moreover, like in fish liver, IGF-I-immunoreactivity could not be localized in rat liver but was detectable only after pretreatment with colchicine (Hansson et al. 1988). Thus, it is reasonable to assume that IGF-I after synthesis is rapidly released from the hepatocytes into the circulation (Reinecke et al. 1997; Plisetskaya 1998).

In the present study, radioimmunoassayable IGF-I in wild-type tilapia liver amounted to about 3.8 ng/g (0.5 pmol/g). Until now, no results have been

reported for fish liver IGF-I measured with a species-specific RIA. A significantly (about fourfold) higher level of IGF-I was found in the liver of transgenics (16.0 ng/g; 2.1 pmol/g). This is paralled by the detection of IGF-I-immunoreactive hepatocytes in transgenic liver which were not observed in wild-type, as well as by the increase in IGF-I mRNA expression as revealed by RT-PCR and in situ hybridization. Because the latter results may also indicate a higher release of IGF-I into the circulation the level of IGF-I in serum was determined. In wild-type individuals it amounted to 15 ng/ml (~2 pmol/ml) whereas in the transgenics it was 6.2 ng/ml (0.82 pmol/ml). Comparison of the serum IGF-I levels published for diverse bony fish shows a broad range. The serum IGF-I concentration in wild-type *O. niloticus* as measured here (15 ng/ml) is lower than that reported in two previous studies on another tilapia species, *O. mossambicus* (150 ng/ml, Riley et al. 2002; 120 ng/ml, Uchida et al. 2003), but consistent with recent data on *O. mossambicus* (~20 ng/ml, Fiess et al. 2007) obtained under similar rearing conditions (28°C, freshwater, feeding) as used here, and on rainbow trout (11.5 ng/ml, Gabillard et al. 2003), brown trout (42.2 ng/ml, Baños et al. 1999), Atlantic (53.1 ng/ml, Dyer et al. 2004) and Coho salmon (7.5–14 ng/ml, Shimizu et al. 1999; 25.9 ng/ml, Shimizu et al. 2000; 45.2–85 ng/ml, Moriyama 1995). In Coho salmon (Shimizu et al. 2003) and gilthead seabream (Mingarro et al. 2002), plasma IGF-I levels changed with the seasons and were higher in fed fish reared in a warm environment (10–15 ng/ml) than in starved fish reared in the cold (5–9 ng/ml, Larsen et al. 2001). In several salmonids, liver IGF-I mRNA and/or plasma IGF-I levels were related to environmental temperature (Gabillard et al. 2003; Larsen et al. 2001; Beckman et al. 1998). The largely varying serum IGF-I levels in bony fish may, thus, be due to species differences but may as well reflect physiological parameters such as nutritional status, seasons and environmental temperature (Reinecke and Collet 1998; Duan 1998; Reinecke et al. 2005).

As a first step towards identifying specific IGF-I binding (IGF binding proteins) in tilapia serum and liver we used incubation with $^{125}$I-IGF-I and subsequent gel filtration at neutral pH (radiochromatography). The main $^{125}$I-IGF-I binding peak of tilapia serum eluted at the same position (40–50 kDa) as in human serum (Zapf et al. 1975). In human serum, this peak contains all six IGFBPs, BP-1 to BP-6 (Zapf 1995). It is likely that the five IGFBPs detected in fish serum so far (Duan and Xu 2005; Shimizu et al. 2005; Kelley et al. 2002, 2006) are also located in this binding complex. Serum of the transgenics bound more tracer than wild-type serum. The height of the binding peak is determined by two factors, the amount of binding protein and its IGF content. Increased binding of $^{125}$I-IGF-I in the transgenic serum could therefore be due to its lower IGF-I concentration resulting in a greater residual binding capacity or to a higher IGFBP concentration. In agreement with the increased binding of $^{125}$I-IGF-I in the serum of the transgenic (GH-overexpressing) tilapia, short-term application of GH has been shown to up-regulate the BP serum levels in Coho salmon (Shimizu et al. 1999; Kelley et al. 1992), striped bass (Siharath et al. 1995) and tilapia (*O. mossambicus*; Park et al. 2000).

Whereas the radiochromatographic pattern of human serum displays an IGF binding peak at 150 kDa (Zapf et al. 1975) consisting of a ternary complex of IGFBP-3, IGF-I and -II, and an acid-labile subunit (ALS, Baxter et al. 1989), the tested tilapia serum showed only a minor binding peak in the 150 kDa region. Whether this peak corresponds to the ternary complex of mammalian serum is not clear. However, no evidence for a ternary complex has so far been obtained in fish serum (Kelley et al. 2006).

In contrast to serum, the protein-bound radioactivity in tilapia liver homogenate eluted as a relatively broad shoulder. It was shifted towards the peak of non-bound tracer and, thus, apparently contained also IGFBPs of smaller molecular weight or IGFBP degradation products. The observed increased binding of tracer in transgenic liver is compatible with a greater binding protein content than in wild-type liver because the IGF-I concentration in the transgenic liver was higher and binding capacity would therefore be expected to be lower.

In GH-overexpressing Coho salmon, IGF-I serum levels varied (Devlin et al. 2000): they were either slightly enhanced or slightly reduced. Our finding that the IGF-I serum levels in the growth-enhanced transgenic tilapia were significantly by 60% lower than in wild-type fish, despite increased IGF-I mRNA expression and peptide levels in liver, was unexpected. This observation appears to be in contrast to

results obtained in *O. mossambicus* demonstrating that body length and mass are positively correlated with the plasma IGF-I concentration (Kajimura et al. 2001; Uchida et al. 2003). Moreover, a clear correlation between individual serum IGF-I levels and growth rates has been established in Coho salmon (Pierce et al. 2001). How could the lower IGF-I serum levels in the transgenic animals be explained? The increased content of IGFBPs in the liver of the transgenics may lead to retention of IGF-I by these BPs. This is further supported by the finding that IGF-I-immunoreactivity was present in hepatocytes of transgenic but not of wild-type liver.

In addition then, the question arises why transgenic tilapia have an approximately 1.5-fold greater body length and a 2.3-fold higher body weight than the wild-type despite lower serum IGF-I. Recent studies using the Cre/loxP recombination system to delete the IGF-I gene exclusively in the liver of mice (Sjögren et al. 1999; Yakar et al. 1999) underline the potential importance of local IGF-I production for growth. Despite deletion of liver IGF-I mRNA expression and largely reduced levels of circulating IGF-I, the animals did not show any obvious impairment of postnatal body growth. Similarly, in the transgenic tilapia, IGF-I in extrahepatic sites may determine the growth rate. This has been shown here to be true for skeletal muscle that is essentially involved in growth. Enhanced growth therefore seems to be caused by higher tissue levels of IGF-I under the influence of the GH transgene and thus by autocrine/paracrine actions of IGF-I.

**Acknowledgements** The authors are grateful to Ms Cornelia Zwimpfer, Division of Endocrinology and Diabetes, Department of Medicine, University Hospital Zürich, for excellent support in the performance of radioimmunoassay and radiochromatography. The study was supported by the Swiss National Science Foundation (grant no. 111028) and the Hartmann Müller-Foundation for Medical Research (grant no. 1115).

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